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Copper resistance and its regulation in the sulfate reducing bacterium *Desulfosporosinus* sp. OT

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Abbreviations: MBD, metal binding domain; Ni-NTA, nickel-nitrilotriacetic acid; EMSA, electrophoretic mobility shift assay; IPTG, isopropyl-β-D-thiogalactoside; ROS, reactive oxygen species; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(N-morpholino)propanesulfonic acid, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodiumdodecyl sulfate; rTEV, recombinant tobacco etch virus; Ec_CopA, *Escherichia coli* CopA; Eh_CopA, *Enterococcus hirae* CopA; DOT_CopA, *Desulfosporosinus* sp. OT CopA; DOT_CopA2, *Desulfosporosinus* sp. OT CopA2.

Desulfosporosinus sp. OT is a Gram-positive, acidophilic sulfate-reducing Firmicute isolated from copper tailings sediment in the Norilsk mining-smelting area in Siberia and represents the first *Desulfosporosinus* species whose genome has been sequenced. *Desulfosporosinus* sp. OT is exceptionally copper resistant, which made it of interest to study the resistance mechanism. It possesses a *copUAZ* operon which is here shown to be involved in copper resistance. The *copU* gene encodes a CsoR-type homotetrameric repressor. By electrophoretic mobility shift assay, it was shown that CopU binds to the operator/promoter region of the *copUAZ* operon in the absence of copper, and is released from the DNA by Cu^{+} or Ag^{+}, implying that CopU regulates the operon in a copper/silver-dependent manner. DOT_CopA is a P1B-type ATPase related to other characterized, bacterial copper ATPases. When expressed in a copper-sensitive *Escherichia coli* Δ*copA* mutant, it restores copper resistance to wild-type levels. His-tagged DOT_CopA was expressed from a plasmid in *E. coli* and purified by Ni-NTA affinity chromatography. The purified enzyme is most active in the presence of Cu(I) and bacterial phospholipids. These findings indicate that the *copUAZ* operon confers copper resistance to *Desulfosporosinus* sp. OT, but do not *per se* explain the basis of the high copper resistance of this strain.

INTRODUCTION

Copper is a cofactor in many bacterial enzymes such as cytochrome aa_3-type terminal oxidases, Cu,Zn-superoxide dismutase required for defense against oxidative stress, plastocyanins and azurins which act as electron carriers, and periplasmic multicopper oxidases which can oxidize Cu^{+} to less toxic Cu^{2+} (Grass *et al.*, 2001; Solioz *et al.*, 2010; Abicht *et al.*, 2013). It is currently believed that copper-loading of all these proteins takes place in the periplasmic space and that there is no requirement for cytoplasmic copper in bacteria, with the exception of photosynthetic organisms (Raimunda *et al.*, 2011). While this concept still awaits more extensive scientific proof, it is clear that excess cytoplasmic copper is toxic and all bacteria are endowed with one or several copper export mechanisms as well as various cytoplasmic copper sequestration and detoxification schemes (Dupont *et al.*, 2011; Bondarczuk and Piotrowska-Seget, 2013; Osman and Cavet, 2008).

The ability of copper to catalyze the formation of reactive oxygen species (ROS) *via* Fenton-type chemistry has frequently been stated to be the primary toxicity mechanism of copper. However, recent work suggests that *in vivo* copper toxicity is primarily due to the displacement of iron from iron-sulfur clusters, leading to the inactivation of essential enzymes (Macomber and Imlay, 2009; Macomber *et al.*, 2007; Chillappagari *et al.*, 2010; Azzouzi *et al.*, 2013; Fung *et al.*, 2013; Foster *et al.*, 2014).

The key components of copper homeostasis in all bacteria are Cu^+-ATPases, usually termed CopA, CopB, or CupA. They belong to the heavy metal-transporting P1B-type ATPases subgroup of the P-type ATPases superfamily (Lutsenko and Kaplan, 1995). Their characteristics are one to six CxxC metal binding domains at their N-termini, and conserved DKTGT (in the one-letter amino acid code, used throughout this publication) phosphorylation domains, ATP-binding domains, and intramembranous CPC or CPH motifs involved in copper transport. The X-ray crystal structure has so far only been solved for CopA of *Legionella pneumophila* (Gourdon *et al.*, 2011). It is now clear that CopA-type ATPases remove excess Cu^+ from the cytoplasm by pumping it across the cytoplasmic membrane.

We recently announced the draft genome of a Gram-positive, sulfate-reducing bacterium, *Desulfosporosinus* sp. OT (Abicht *et al.*, 2011). *Desulfosporosinus* bacteria were identified as key players in microbial sulfate reduction in a low-sulfate peatland. Sulfate reduction contributes to precipitation of metal sulfides and thereby to the immobilization of toxic metals (White *et al.*, 1997). In culture, *Desulfosporosinus* sp. OT exhibits unusually high copper tolerance, being able to grow in 236 mM copper under sulfate reducing conditions (Abicht *et al.*, 2011).

Copper homeostasis has been well characterized in two other Gram-positive organisms, namely *Lactococcus lactis* and *Enterococcus hirae* (Solioz *et al.*, 2010). In *L. lactis*, the core element of copper resistance is the *copRZA* operon, which encodes the CopR copper-inducible repressor, the CopZ copper chaperone and the CopA copper export ATPase (Magnani *et al.*, 2008). Copper activates the operon by inducing the release of the CopR repressor from the *copRZA* operator-promoter (Portmann *et al.*, 2006). A second putative copper ATPase on a monocistronic operon, CopB, is also under the control of CopR; however, its role remains unclear (Magnani *et al.*, 2008). Likewise, the copper resistance determinant of *E. hirae* is the *copYZAB* operon. It is under the control of the copper-inducible repressor CopY. Of the two ATPases, CopA and CopB, only CopB appears to contribute to copper resistance. CopA, like CopB of *L. lactis*, has recently been proposed to have a role in the supply of copper to the periplasm for copper-loading of enzymes (Raimunda *et al.*, 2011).

The genome of *Desulfosporosinus* sp. OT encodes two putative copper exporting ATPases, CopA and CopA2. In this work, the determinant of copper resistance was shown to be the *copUAZ* operon, encoding a copper-responsive inducer, CupU, which regulates the operon, a copper-exporting ATPase, CopA, and a putative cytoplasmic copper chaperone, CopZ. No role in copper resistance could be defined for CopA2.

METHODS

Reagents and chemicals. All reagents were of analytical grade and were obtained from Sigma-Aldrich if not indicated differently. Ni-NTA affinity resins were from Qiagen and asolectin from Associated Concentrates, Woodside, NY. *E. coli* and *E. hirae* lipids were isolated as previously described (Ames, 1968).

Strains and culture conditions. *Desulfosporosinus* sp. OT was grown as previously described (Karnachuk *et al.*, 2005) and DNA was isolated by alkaline lysis as described elsewhere (Ausubel *et al.*, 1995). *E. coli* W3110 and W3110Δ*copA* were kindly provided by Christopher Rensing, University of Copenhagen. All *E. coli* strains were grown aerobically in LB media at 37 °C, unless indicated differently.

Vector construction. The *E. coli* CopA gene (*Ec_copA*) was cloned by PCR amplification from *E. coli* W3110 genomic DNA with primers ha95 and ha96 (Table S1). The resulting PCR product was cloned into PCR Blunt II TOPO (Invitrogen, California, USA), yielding pCA6. The *Ec_copA* gene was excised from pCA6 with NarI and XbaI and subcloned into pProExHTa, digested with the same enzymes. The resulting construct, pCB1, encoded Ec_CopA with an N-terminal His-tag that could be cleaved with recombinant tobacco etch virus (rTEV) protease. *Desulfosporosinus* sp. OT *copU* (NCBI accession: AGAF01000118) was cloned by PCR amplification with *Pfu* DNA polymerase using primers U1 and U2. The PCR product was cloned into pCR-Blunt II-TOPO, generating pOT07, from where the *copU* gene was excised with NarI and XbaI and subcloned into pProExHTa, digested with the same enzymes. The resulting construct, pOU02, encoded CopU with an rTEV-cleavable, N-terminal 6-His-tag. *DOT_copA* (NCBI accession number: EGW37486) was similarly cloned using PCR primers ha91 and ha92 for cloning into PCR Blunt II TOPO, yielding pOT5, followed by subcloning into pProExHTa. The resulting construct, pOU1, encoded DOT_CopA with an rTEV-cleavable, N-terminal 6-His-tag. *DOT_copA2* (NCBI accession: AGAF01000248) was cloned by the same strategy, using PCR primers A21 and A22 to generate pOT91, from which the gene was subcloned into pProExHTa to generate pOU03, encoding DOT_CopA2 with an rTEV-cleavable, N-terminal 6-His-tag. The absence of mutations was verified in all the constructs by commercial DNA sequencing.

Purification of CopU. BL21(DE3)RIL with plasmid pOU02 was grown aerobically at 37 °C in 300 ml of LB containing 50 μg/ml of ampicillin to an OD at 600 nm of 0.8. The culture was then induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated for additional 16 h at 20 °C. The cells were harvested at 7000 x g for 10 min and the resultant pellet was stored at –70^{o}C until further use. His-tagged CopU was purified by re-suspending

pelleted cells in 2 ml per g of wet weight in lysis buffer (50 mM Na-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5), containing 1 mM phenylmethylsulfonyl fluoride (PMSF), added from 100 mM stock in dimethylsulfoxide. Cells were disrupted by two passages through a French press at 30 MPa. Cell debris was removed by centrifugation at 15,000 x g for 30 min. The supernatant obtained was applied to a Ni-nitrilotriacetic acid (Ni-NTA) column equilibrated with lysis buffer. The column was washed with 5 column volumes each of lysis buffer, lysis buffer with 25 mM imidazole, and lysis buffer with 50 mM imidazole. CopU was eluted with lysis buffer containing 200 mM imidazole. Eluted fractions were analyzed n 15% sodiumdodecyl sulfate (SDS) polyacrylamide gels (Laemmli, 1970). CopU-containing fractions were pooled and dialyzed against 2 x 100 volumes of 20 mM Na-HEPES pH 7.5, 1 mM dithiothreitol, for 2 h each. The His-tag of CopU was removed by cleavage with 1/10 the amount of rTEV protease (purified in-house) overnight at room temperature in the same buffer, followed by passage through a Ni-NTA column to remove uncleaved His-CopU and the His-tagged rTEV protease. The resulting 115 amino acid-protein with a predicted molecular weight of 13 kDa exhibited high purity (> 95%), as determined by SDS polyacrylamide gel electrophoresis (Fig. S1). Purified CopU was incubated with 1/20 volume of Chelex 100 (Bio-Rad, Berkeley, USA) for 2 h at 4 °C to remove metal ions, followed by dialysis against 2 x 100 volumes of 20 mM Na-HEPES pH, 1mM dithiothreitol, for 2 h each at 4 °C. The protein concentration was determined by the method of Bradford, using bovine serum albumin as a standard (Bradford, 1976).

Crosslinking of CopU. Purified CopU (5 µg) was suspended in 50 mM Na-HEPES pH 7.5 and crosslinked for 5 to 20 min with 0.2% glutaraldehyde in a total reaction volume of 25 µl. Reactions were stopped by the addition of 5 µl of 1 M Tris-Cl pH 8 and crosslinking was evaluated by electrophoresis on a 10% SDS-polyacrylamide gel, followed by staining with Coomassie blue.

Electrophoretic mobility shift assays (EMSA). Purified CopU and DNA were incubated at molar ratios of 240:1 and incubated for 1 h at room temperature in a total volume of 20 µl of binding buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA, 100 mM KCl, 200 µM Mg-acetate, 100 µM dithiothreitol, 1 µg bovine serum albumin, 5% glycerol). Samples were separated on 10% polyacrylamide gels prepared in a 1:1 mixture of binding buffer and 40 mM Tris-acetate pH 8, 1 mM EDTA. Following electrophoresis, gels were stained for 60 min with RedSafe (Sigma-Aldrich, St. Louis, MO, USA), diluted 1:20'000 in 40 mM Tris-acetate pH 8, and photographed under blue LED illumination with a Bio-Rad ChemiDoc imaging system. Primers ms111/ms112 and ms115/ms116 were pairwise annealed at 65 °C for 10 min for

EMSA. PCR products were amplified with primer pairs sm144/ms110 and ms109/sm143 (Table S1) from *Desulfosporosinus* sp. OT DNA with *Pfu* DNA polymerase under standard conditions. Identical EMSA results were obtained with His-tagged CopU and CopU without the His-tag.

Purification of DOT_CopA and DOT_CopA2. The same procedure was used both, for DOT_CopA and DOT_CopA. *E. coli* BL21(DE3)RIL transformed with either plasmid pOU1 or pOU3 was grown aerobically in 1 l (2 x 0.5 l in baffled 1 l Erlenmeyer flasks) of 1% Trypticase-peptone, 1% yeast extract, 0.5% NaCl, at 37 °C to an OD at 600 nm of 0.5–0.7. Expression was then induced with 0.1 mM IPTG and the cultures grown for an additional 4 h. Cells were collected by centrifugation for 10 min at 8000 x g at room temperature and washed with 250 ml of 0.9% NaCl. They were finally resuspended in 10 ml per g of wet pellet of G-buffer (20 mM Tris-SO_4 pH 7.5, 5 mM $MgSO_4$, 25 mM Na_2SO_4, 25 mM K_2SO_4, 1 mM β-mercaptoethanol, 20% (v/v) glycerol, 5 µg/ml DNaseI, 1 mM PMSF) and passed through a French press twice at 30 MPa. Unbroken cells were removed by centrifugation for 15 min at 12,000 x g and the supernatant was centrifuged for 45 min at 90,000 x g. The resultant membrane pellet was resuspended in 2.5 ml of G-buffer and stored frozen at -70°C. For ATPase purification, the membranes were suspended in G-buffer at a protein concentration of 24 mg/ml, supplemented with 1/100 volume of a protease inhibitor cocktail (100 mM N-α-*p*-tosyl-L-lysine-chloromethylketone, 100 mM N-*p*-tosyl-L-phenylalanine-chloromethylketone, 100 mM *p*-aminobenzamidine, 100 mM phenylmethylsulfonyl fluoride dissolved in dimethylsulfoxide), and extracted with dodecyl-β-D-maltoside at a detergent/protein ratio of 8 (w/w) with stirring on ice for 1 h. Undissolved membranes were sedimented for 45 min at 90,000 x g and the supernatant loaded onto a 1 ml Ni-NTA-agarose column, pre-equilibrated with buffer JD (20 mM Tris-SO_4 pH 7.5, 5 mM $MgSO_4$, 1 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.05% dodecyl-β-D-maltoside). Weakly bound proteins were washed from the column with buffer JD buffer containing 10 mM of imidazole. Elution of the ATPase was accomplished with a 30 ml linear gradient of 10–250 mM imidazole in buffer JD. The eluate was desalted on a PD10 column (GE Healthcare Life Sciences) equilibrated with buffer JD. Cleavage of the 6 His-tag was accomplished as described above for CopU. Purified ATPase was stored at -70 °C. The yield was typically 1 mg of ATPase.

Complementation of *E. coli*. The *E. coli* W3110Δ*copA* strain was transformed with either the empty vector or constructs pCB01, pOU1 or pOU3, expressing Ec_CopA, DOT_CopA, or DOT_CopA2, respectively. Cultures were grown in LB media containing 50 µg/ml of ampicillin and no copper, 1.5 or 3 mM $CuSO_4$ and the optical density was determined at 600

nm after 48 h of aerobic growth at 37 °C. No IPTG was added to the cultures, since it had a strong inhibitory effect on growth, presumably due to excessive expression of the ATPases compared to wild-type levels. For complementation studies on solid media, 100 µl of exponential-phase cultures were suspended in 2.5 ml of 0.7% agar and poured on LB plates. Filter disks containing 5 µl of 1 M $CuSO_4$ were deposited on the plates and after 24 h of incubation at 37 °C, the plates were photographed with a digital camera.

ATPase activity measurements. Enzyme activity was measured at 37 °C in a volume of 1 ml 40 mM Na-3-(N-morpholino)propanesulfonic acid (MOPS), pH 6.5, 150 mM NaCl, 20 mM NH_4SO_4, 20 mM L-cysteine, 5 mM KCl, 5 mM $MgCl_2$, 2 mg/ml dodecylmaltoside, 100 µM $CuSO_4$, and supplemented with different lipids as indicated under Results. The reaction was started by the addition of 1 mM Na-ATP and was followed my measuring Pi-release by the method of Lanzetta et al. (Lanzetta *et al.*, 1979).

RESULTS

Structure of the *copUAZ* operon

Desulfosporosinus sp. OT contains a predicted *copUAZ* operon, encoding a copper-responsive repressor, CopU, a P1B-type ATPase, DOT_CopA, and a CopZ copper chaperone (Fig. 1a). Downstream of *copZ* are two more open reading frames, *orf1* and *orf2*, encoding proteins of unknown function. Whether *orf1* and *orf2* are part of the *cop*-operon currently remains unclear and the operon will henceforth only be referred to as the *copUAZ* operon. The *copUAZ-orf1-orf2* gene cluster is braced by terminators with predicted stabilities of -11.4 and -16.9 kcal. Upstream of *copU* are predicted -10 and -35 regions, and a dyad symmetry with sequence TATAGTATA(N_6)TATACTATA, which could represent the CopU binding site (Fig. 1b). Also shown in this Figure are the oligonucleotides used to test CopU-DNA interaction.

Primary and quaternary structure of CopU. The predicted gene product of *copU* exhibits extensive sequence similarity to characterized CsoR-type repressors (Fig. 2). This type of repressor has been characterized in great detail in recent years and a number of structures have been solved (Higgins and Giedroc, 2014). They reveal a flat, disc-like homotetrameric structure; two such tetramers form a "sandwich" complex with the operator DNA region in the absence of copper to suppress transcription (Chang *et al.*, 2015). In CsoR of *Mycobacterium tuberculosis*, Cu(I) binds to C36 on one subunit and H61 and C65 on the adjacent subunit (Liu *et al.*, 2007). Furthermore, Y35 and E81 were shown to be involved in a hydrogen bonding network between the subunits (Higgins and Giedroc, 2014; Chang *et al.*,

2015). All these amino acids are conserved in CopU, suggesting a similar regulatory mechanism.

Structural modeling of CopU predicts a homotetrameric structure that is essentially identical to that of other CsoR-type repressors (Fig. S2) (Porto *et al.*, 2015; Jacobs *et al.*, 2015; Chang *et al.*, 2014; Liu *et al.*, 2007). The subunit composition of purified CopU (Fig. S1) was verified experimentally by crosslinking of the purified protein with glutaraldehyde. The subunits of multimeric proteins in suspension can readily be crosslinked with bifunctional crosslinking reagents, while monomeric proteins in solution do not significantly crosslink under similar conditions. Crosslinking CopU with glutaraldehyde led to the formation of dimers, trimers, and tetramers of the expected molecular weights of 26, 39, and 52 kDa, but not to higher-order structures (Fig. 3). This suggests that CopU is indeed a homotetramer.

CopU-DNA interaction

Sequence analysis of the promoter/operator region revealed a 9-bp inverted repeat at position -22 to -45 that likely constitutes a repressor binding site. An 80 bp (ms115/ms116) double stranded oligonucleotide, encompassing this region (cf. Fig. 1b), was tested for interaction with CopU by EMSA. Fig. 4a shows that CopU forms a complex with this DNA region in the absence of copper. Cu^{+} and Ag^{+}, but not Zn^{2+}, Co^{2+}, Cd^{2+}, or Ni^{2+}, dissociate the complex. Silver induction of CopU supports Cu^{+} rather than Cu^{2+} as inducer: Ag^{+} is chemically similar to Cu^{+} but not Cu^{2+} (Outten *et al.*, 2001; Migocka *et al.*, 2015) and Ag^{+} is a known mimetic of copper; induction by Ag^{+} has been shown for many copper-responsive repressors (Odermatt *et al.*, 1993; Rensing *et al.*, 2000; Liu *et al.*, 2007; Magnani *et al.*, 2008). Titration of the CopU-DNA interaction with Cu^{+} showed a gradual dissociation of the complex, with half-maximal release occurring at 10 µM Cu^{+} (Fig. 4b).

A 32 bp DNA douplex (oligonucleotide dimer ms111/ms112), encompassing the *copUAZ* promoter region from position -22 to -45, was apparently too short to form a DNA-CopU complex (not shown, see discussion). A PCR product generated with primers sm144 and ms110, covering nucleotides -96 to -273 and not containing the proposed CopU binding site but including the inverted repeat of the predicted upstream terminator, did also not show CopU-DNA interaction (Fig. 4c). On the other hand, a PCR product of similar size, generated with primers ms109 and sm143, but encompassing nucleotides -145 to +30 and containing the proposed CopU binding site, exhibited copper-dependent CopU binding.

A homotetrameric DNA binding protein must necessarily bind to an inverted repeat sequence for symmetry reasons. There can thus be little doubt that CopU binds to the inverted repeat TATAGTATAgggggTATACTATA, indicated in Fig. 1b**.** Taken together, these

results show that CopU binds to the promoter region of the *copUAZ* operon in a copper-dependent manner and suggest that CopU regulates the expression of the downstream genes, thereby regulating intracellular copper homeostasis in *Desulfosporosinus* sp. OT.

Primary structure of the DOT_CopA ATPase

In the annotated genome of *Desulfosporosinus* sp. OT, two genes were predicted to code for copper ATPases: *DOT_copA*, which is part of the *copUAZ*-operon, and *DOT_copA2*, which is located elsewhere in the genome (Abicht *et al.*, 2011). Both genes code for proteins which strongly resemble the experimentally characterized Ec_CopA of *Escherichia coli* and Eh_CopA of *Enterococcus hirae* and possess the conserved elements typical of copper ATPases (Fig. S3) (Solioz *et al.*, 1994).

A signature feature of heavy metal ATPases is a conserved proline residue in membrane helix six, usually in the context CPC. This site has been shown to directly be involved in transport of Cu^{+} ions across the membrane (Mandal *et al.*, 2004). The amino acids surrounding the conserved proline confer metal specificity to the ATPase. The universal DKTGT motif encompasses the aspartic acid residue which is phosphorylated during catalysis. Other conserved features typical of P1B-ATPases are also conserved in DOT_CopA and DOT_CopA2. These are the TEGS motif, the HP-motif, the TGDN motif, and the GDGINDAPAL motif (see ref. (Smith *et al.*, 2014) for recent review). The presence of these motifs in DOT_CopA and DOT_CopA suggests that both enzymes are heavy metal ion translocating ATPases of P1B-type.

Complementation of *E. coli* with DOT_CopA and DOT_CopA2

To functionally characterize DOT_CopA and DOT_CopA2, the respective genes were cloned in the pProExHTa expression vector and transformed into the copper sensitive *E. coli* W3110Δ*copA* strain, which is devoid of the single transport system, CopA, which can expel cytoplasmic copper (Rensing *et al.*, 2000). Complementation studies were performed both in liquid (Fig. 5a) and on solid media (Fig. 5b) in the presence and absence of copper. As expected, *E. coli* CopA used as a positive control, complemented the copper sensitive phenotype of W3110Δ*copA* in liquid culture as well as on solid media. DOT_CopA also complemented the copper-sensitive phenotype under both conditions, indicating that this enzyme functions as a copper-exporting ATPase in *E. coli*. However, DOT_CopA2 was unable to restore the copper sensitive phenotype of the host strain, suggesting that only DOT_CopA, but not DOT_CopA2, functions in copper resistance of *Desulfosporosinus* sp. OT.

In vitro activity of purified DOT_CopA

To confirm the function of DOT_CopA as a copper ATPase, the enzyme was purified to greater than 90% purity by Ni-NTA affinity chromatography (Fig. S4). ATPase activity by DOT_CopA was highest in the presence of 100 μM Cu^{+}, 20 mM L-cysteine, and 1 mg/ml of phospholipids. Different lipid preparations were tested for their effect on ATPase activity. Asolectin, a commercial crude soy bean phospholipid preparation, was previously found to optimally stimulate different ATPases (Portmann and Solioz, 2005; Wunderli-Ye and Solioz, 2001; Wyler-Duda and Solioz, 1996; Apell and Solioz, 1990). With asolectin, the specific ATPase activity of DOT_CopA was 12 ± 5 nmol/min/mg, depending on the preparation. In the presence of *E. coli* or *E. hirae* phospholipids in lieu of asolectin, the activity was 1.5 to 2.5 times and 2.5 to 3.5 times higher, respectively (Fig. 6). No enzymatic activity could be measured for DOT_CopA2 (not shown). Taken together, the structural features of DOT_CopA, its regulation by the copper-responsive CopU repressor, the complementation of a copper-sensitive *E. coli* phenotype by DOT_CopA, and the *in vitro* ATPase activity strongly suggest that DOT_CopA functions as a copper exporting ATPase in *Desulfosporosinus* sp. OT.

DISCUSSION

Desulfosporosinus OT is a sulfate reducing bacterium that can withstand high ambient copper concentrations (Abicht *et al.*, 2011). To learn about the basis of copper resistance by this organism, the putative copper homeostatic genes/operons *copUAZ* and *copA2* were cloned and expressed in *E. coli* and purified for functional analysis. The *copUAZ* operon, but not *DOT_copA2*, could be shown to have a function in copper resistance.

The fist gene of the *copUAZ* operon, *copU*, encodes a CsoR-type copper-responsive repressor, closely related to CsoR of *Geobacillus thermodenitrificans* (Chang *et al.*, 2015). Interestingly, the 25 N-terminal residues of CopU, which are not seen in the structural model shown in Fig. S2, feature a CxxC motif; this motif is a ubiquitous Cu(I) binding site in copper chaperones and the N-termini of copper ATPases (Boal and Rosenzweig, 2009). In GenBank, this motif is only found in CsoR-type proteins of sulfate reducing bacteria closely related to the one under study here. The Cu(I)-bound structure of CsoR from *Geobacillus thermodenitrificans* reveals that the N-terminus is folded over the Cu(I) binding sites (Chang *et al.*, 2014). The N-terminal CxxC motif of CopU could conceivably participate in copper binding or serve as a docking point for copper chaperones. Why this CxxC feature is only found in acidophilic sulfate reducing bacteria remains open to speculation.

CsoR-type repressors are all-helical, homotetrameric disc-like structures. In the absence of copper, two CsoR tetramers clutch the operator region to prevent transcription (Chaplin *et al.*, 2015). For induction of transcription by copper, four Cu^{+} per homotetramer bind cooperatively to a C and a H residue on one subunit and a C residue of the adjacent subunit (Jacobs *et al.*, 2015). This allosterically reduces or inhibits DNA binding, allowing transcription of the downstream genes to proceed (Chang *et al.*, 2015).

The putative DNA binding site of CopU, TATAGTATAGGGGGGTATACTATA, encompasses a 9-bp inverted repeat (underlined), separated by 6 G-residues. This surmised CopU DNA binding site differs from those of other CsoR-type repressors. CsoR of *G. thermodenitrificans* binds to the minimal motif TACCCCCTTCGGGTA (Chang *et al.*, 2015), while the 'CsoR box' of *Corynebacterium glutamicum* features the sequence ATACCCCTAGGGGGTAT and *Bacillus subtilis* CsoR binds to TACCCTACGGGGGTATGGTA (Teramoto *et al.*, 2012; Smaldone and Helmann, 2007). So the DNA binding sites for CsoR-type repressors, including some not specifically mentioned here, appear to be diverse. The observation that a 32 bp DNA fragment did not support CopU binding *in vitro* suggests that the DNA region occupied by CopU is larger, or could even indicate that four CopU tetramers bind to the operator/promoter region. Indeed, the observed changes in electrophoretic mobility in EMSA experiments appeared very large, although this is inherently difficult to quantify. The promoter region of DOT_CopA2, for which we could not identify a role in copper resistance, does not exhibit any sequence features that would suggest a CopU binding site.

DOT_CopA, which apparently functions as a copper exporter, features three N-terminal CxxC consensus copper binding motifs, one being located in a 26-amino acid domain not present in the other ATPases shown in Fig. S3 or other bacterial copper ATPases. An additional 21-amino acid insertion is present 44 amino acids further downstream of the CxxC-containing insertion. CxxC motifs have been shown to be modular structural elements at the N-termini of copper ATPases. Each module, or metal binding domain (MBD), encompasses around 70 amino acids that are folded in thioredoxin-like fold and can coordinate one Cu^{+} by means of the CxxC motif (Arguello and Gonzalez-Guerrero, 2008; Lutsenko *et al.*, 2007). Bacterial copper ATPases generally feature one (Eh_CopA) or two (Ec_CopA, 2) MBDs, while eukaryotic copper ATPases may possess up to six MBDs. Structure predictions suggest that all three CxxC motifs of DOT_CopA exhibit the typical MBD-fold. For bacterial copper ATPases, the MBDs have been shown to be dispensable, at least under laboratory conditions (Arguello *et al.*, 2007; Fan *et al.*, 2001). It is notable that both, CopU as well as DOT_CopA,

feature additional CxxC motifs not present in analogous proteins of non-acidophilic organisms. *Desulfosporosinus* sp. OT can be cultivated in up to 236 mM copper under sulfate reducing conditions. It could be speculated that the additional CxxC motifs are an adaption to high copper concentrations and/or the acidic environments these bacteria normally live in. However, it must be considered that growth under sulfate reducing conditions leads to the release of hydrogen sulfide, which precipitates heavy metal ions in the environment as insoluble metal sulfides. This can dramatically lower the free, or bioavailable copper concentration. Given the genomic analysis and the structure and function of the *copUAZ* operon characterized here, it appears likely that *Desulfosporosinus* sp. OT is not endowed with an exceptional copper resistance system, but relies on metal sulfide precipitation as a major mechanism to lower the concentration of toxic heavy metals in the environment. Indeed, efficient metal sulfide precipitation from acid mine drainage by sulfate reducing bacteria has been demonstrated in experimental systems (Webb *et al.*, 1998). Taken together, the present work suggests that the *copUAZ* operon constitutes the major copper resistance determinant of *Desulfosporosinus* sp. OT. The only other putative copper ATPase encoded by the genome, DOT_CopA2, could not complement a copper-sensitive *E. coli* strain and did not exhibit copper-stimulated ATPase *in vitro*. DOT_CopA is under the control of a CsoR-type, copper-responsive repressor, CopU. This repressor features an N-terminal CxxC motif, not present in other characterized CsoR-type repressors, an aspect that deserves further investigation.

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FIGURE LEGENDS

Fig. 1. *CopUAZ* operon and promoter structure. (a) *CopUAZ* operon, showing the gene arrangement and predicted proteins with the number of amino acids (open arrows). The black boxes indicate predicted terminators (term) of calculated stability -11.4 kcal and -16.9 kcal. The scale gives distances in base pairs (bp). (b) Promoter/operator region of the *copUAZ* operon. The open arrows indicate the position of a predicted terminator (term), the arrows the location of the putative CopU binding dyad, and the dashed double-lines the primers used for EMSA. The boxes delineate the "-10" and "-35" regions. The ribosome binding site is underlined and *Met-* indicates the first amino acid of CopU. The numbers below the sequence give the nucleotide positions relative to the start of translation.

Fig. 2. Alignment of CsoR-type repressors. The alignment shows, with locus tags in parenthesis: Bsu_CsoR, *Bacillus subtilis* CsoR (BSU33520); DOT_CopU, *Desulfovibrio* sp. OT CopU (WP_009622713), Gth_CsoR, *Geobacillus thermodenitrificans* CsoR (GTNG_1533); Mtu_CsoR, *Mycobacterium tuberculosis* CsoR (Rv0967); Mtu_RicR, *Mycobacterium tuberculosis* RicR (MT0200); Sli_CsoR, *Streptomyces lividans* (SCO4136). The conserved copper-binding Cys and His residues are boxed, with position numbers corresponding to Mtu_CsoR. Also boxed are Tyr-35 and Glu-81, which participate in the hydrogen bonding network between subunits. The CxxC motif of DOT_CopU is underlined.

Fig. 3. Cross-linking of CopU. Aliquots of 5 μg of purified CopU with the his-tag removed were crosslinked with 0.2% glutaraldehyde for the times indicated in the Figure, followed by SDS polyacrylamide gel electrophoresis and staining with Coomassie blue. Std, molecular weight standards of the sizes indicated in kDa on the left side of the gel. The scale on the right indicates monomers (1), dimers (2), timers (3) and tetramers (4).

Fig. 4. EMSA with CopU. (a) The oligonucleotide dimer ms115/ms116(1 pmol), covering bp -85 to -5 of the *copUAZ* promoter and containing the putative CopU binding dyad, was interacted with purified CopU (240 pmol) without metal ions (Ctrl) or 10 μM of the metal ions indicated in the Figure. (b) EMSA in the absence (-) or presence of increasing concentrations of Cu^{+} as indicated in the Figure. Ctrl, no CopU added. (c) EMSA with 1 pmol of PCR product generated with primers sm144/ms110, covering bp -96 to -273, not containing the CopU binding dyad, and ms109/sm143, covering bp -145 to +30 and containing the CopU binding dyad of the *copUAZ* promoter. Ctrl, DNA alone; -, plus 240 pmol CopU; Cu^{+}, plus 240 pmol CopU and 10 μM Cu^{+}. The arrows indicate the migration of free DNA and the asterisks that of the DNA-CopU complex. Other details are given under Methods.

Fig. 5. Complementation of *E. coli.* (a) The growth response to copper in LB media was compared between *E. coli* wild-type (●), the copper-sensitive *E. coli* Δ*copA* mutant (○), and *E. coli* ΔcopA, complemented with either a control vector (□), or a vector expressing Ec_CopA (■), DOT_CopA (▲), or DOT_CopA2 (Δ). Cultures were challenged with the indicated $CuSO_4$ concentrations and grown aerobically for 48 h at 37 °C, followed by measurement of the absorption at 600 nm. The Figure is representative of three independent replicates. (b) Filter disks soaked with 5 μl of 1 M $CuSO_4$ were deposited on bacterial lawns of either wild-type *E. coli* (Wild-type), or an *E. coli* Δ*copA* mutant (Δ*copA*), which was untransformed (-) or transformed with an empty control vector (Vector), or with plasmids harboring the ATPase genes indicated in the Figure. Following incubation for 24 h at 37 °C, the plates were photographed.

Fig. 6. I*n vitro* ATPase activity of purified DOT_ATPase with different lipids. ATPase activity of purified CopA in the presence of 1 mg/ml of different lipids was determined by measuring the release of Pi from ATP. The following lipids were tested: asolectin (○), *E. coli* phospholipids (●), and *E. hirae* phospholipids (▲). Other details of the experiment are described under Methods. The Figure shows one of three independent experiments.

Fig. 1

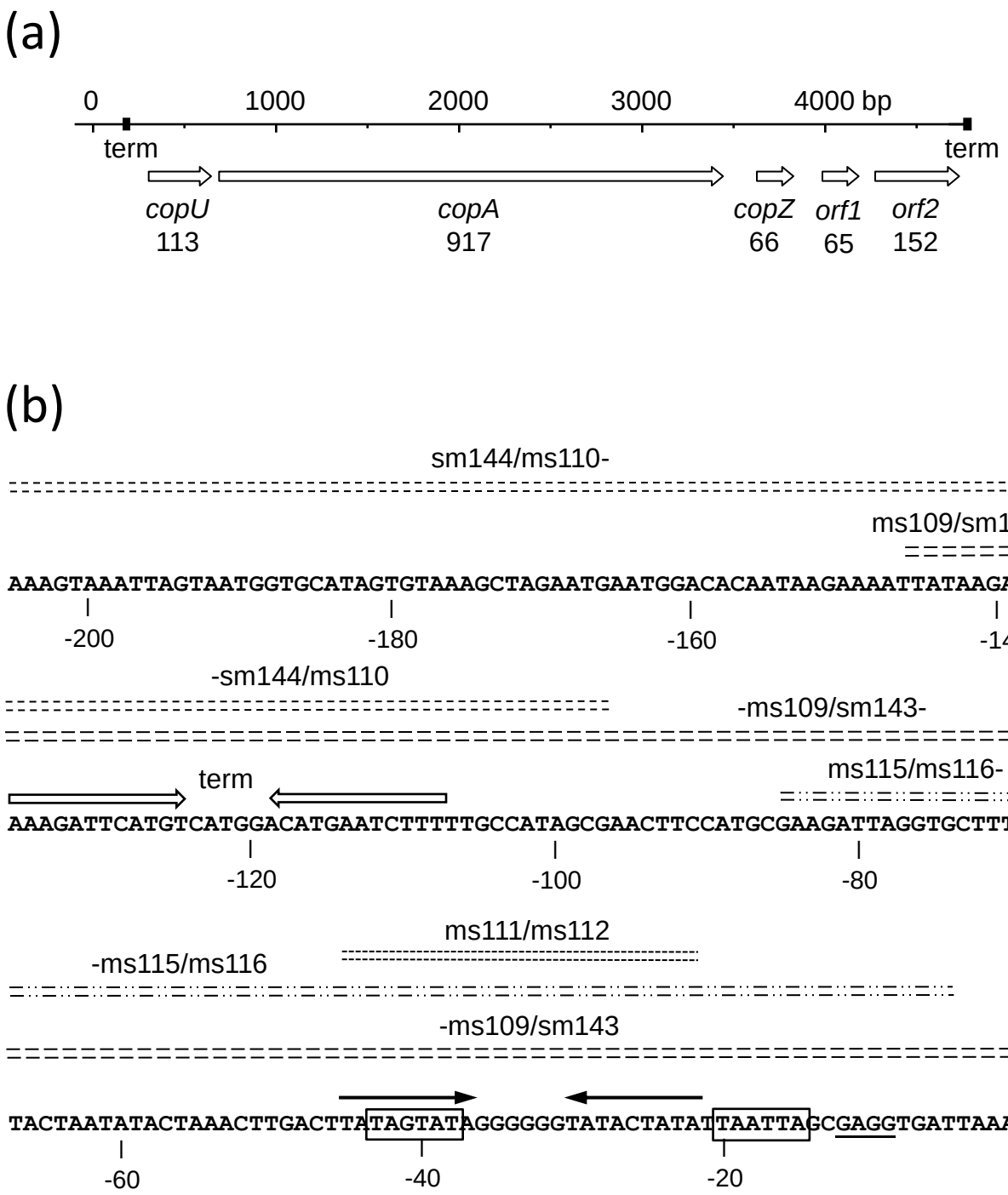

(a)
0
1000
2000
3000
4000 bp
term
term
copU
113
copA
917
copZ
66
orf1
65
orf2
152
(b)
sm144/ms110-
ms109/sm143-
AAAGTAAATTAGTAATGGTGCATAGTGTAAAGCTAGAATGAATGGACACAATAAGAAAATTATAAGATG-
-200
-180
-160
-140
-sm144/ms110
-ms109/sm143-
term
ms115/ms116-
AAAGATTCATGTCATGGACATGAATCTTTTTGCCATAGCGAACTTCCATGCGAAGATTAGGTGCTTTAA-
-120
-100
-80
ms111/ms112
-ms115/ms116
-ms109/sm143
Met-
+1
TACTAATATACTAAACTTGACTTATAGTATAGGGGGGTATACTATATTAATTAGCGAGGTGATTAAATGA
-60
-40
-20

Fig. 2

```
                                .         .         .         .         .
Bsu_CsoR     1> MEKHNE--------------HKTLNHKSSKEKDQITNRLKRIEGQVRGIQ
DOT_CopU     1> MNSEEKVHSCSLCQSDGENQGERTSHHDDKTIKELVTRMNRIEGQIRGIK
Gth_CsoR     1> MTHPSQ---------EEHVLHGTMIPRTKEEIENIMKRLKRIEGQVRGVQ
Mtu_CsoR     1> MSK-----------------------ELTAKKRAALNRLKTVRGHLDGIV
Mtu_RicR     1> MT---------------------AAHGYTQQKDNYAKRLRRVEGQVRGIA
Sli_CsoR    17> GAVNQTVRQAETDGTDIVTDHDRGVHGYHKQKAEHLKRLRRIEGQIRGLQ
                      36.         .         .  61  65 .         .
Bsu_CsoR    37> NMVENDRYCVDILVQISAVQAAMKNVALHLLEDHAHHCVADAIKSGDGE-
DOT_CopU    51> GMIERHVYCDDVLNQIASAQSALDGAARLLLEKHMKSCVKEQLQAGD-E-
Gth_CsoR    42> KMVEDNRYCIDILVQISAIQAALRQVGMQLLERHANHCVAKAIREGSGE-
Mtu_CsoR    28> RMLESDAYCVDVMKQISAVQSSLERANRVMLHNHLETCFSTAVLDGHGQ-
MtuRicR     30> RMIEEDKYCIDVLTQISAVTSALRSVALNLLDEHLSHCVTRAVAEGGPGA
Sli_CsoR    51> RMVDEDVYCIDILTQVSASTKALQSFALQLLEEHLRHCVADAALKGGTEI
                  81    .         .         .         .         .
Bsu_CsoR    86> -QAISELLDVFKKFTKS
DOT_CopU    99> -QVVDEVLKTIFRM-IR
Gth_CsoR    91> -QSLRELMDVIKQF-AK
Mtu_CsoR    77> -AAIEELIDAVKFTPALTGPHARLGGAAVGESATEEPMPDASNM
MtuRicR     80> DGKLAEASAAIARLVRS
Sli_CsoR   101> DAKVEEATKAIGRLLRT
```

Fig. 3

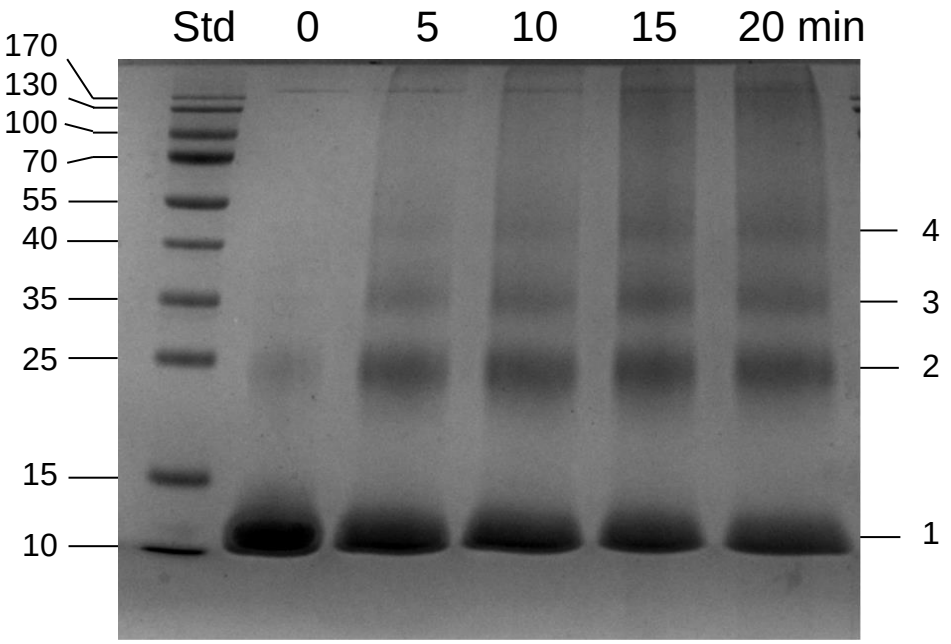
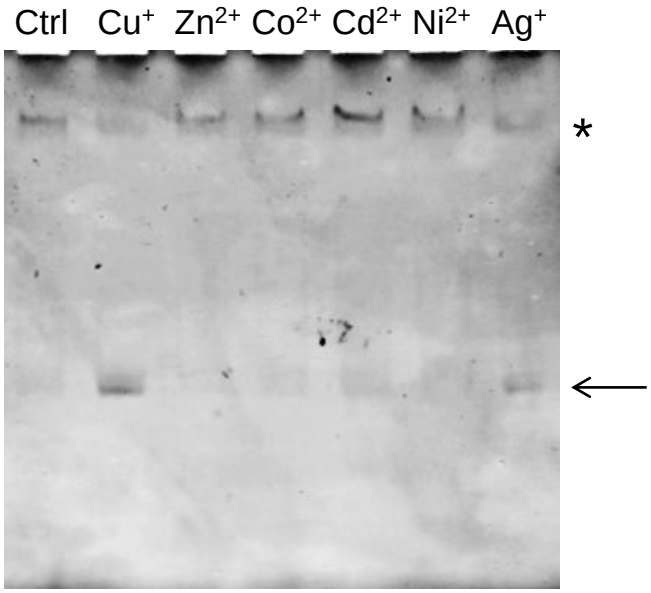
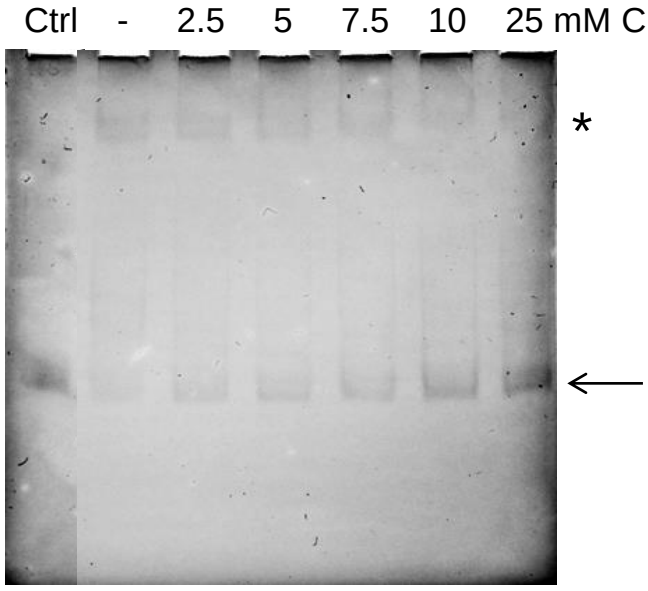


Fig. 4

(a)


Ctrl
Cu+
Zn2+
Co2+
Cd2+
Ni2+
Ag+
*

(b)


Ctrl
-
2.5
5
7.5
10
25 mM Cu+
*

(c)

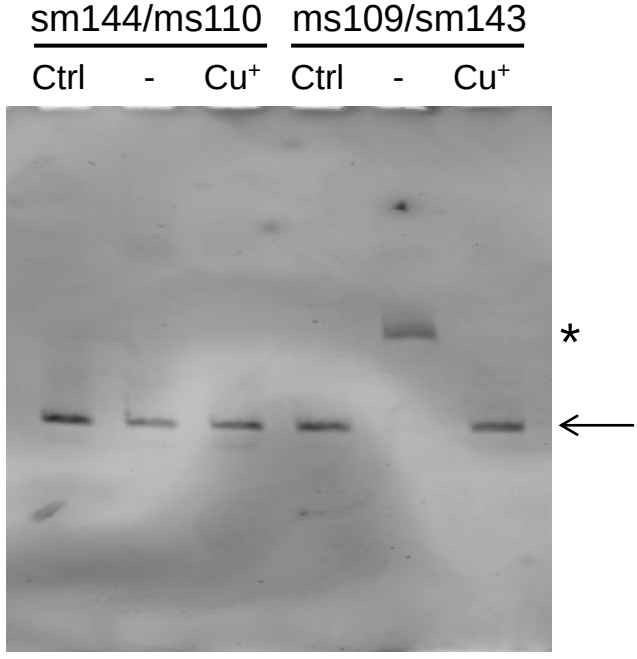

sm144/ms110
ms109/sm143
Ctrl
-
Cu+
Ctrl
-
Cu+
*

Fig. 5

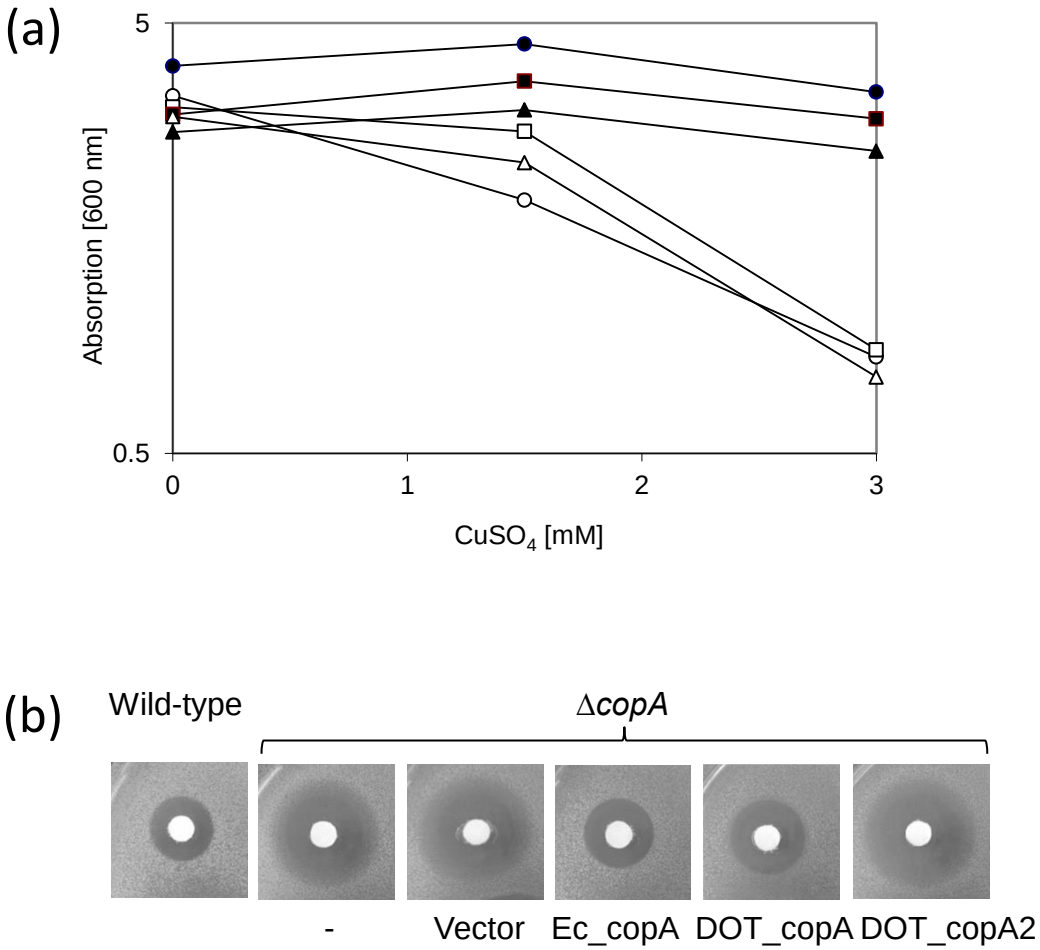
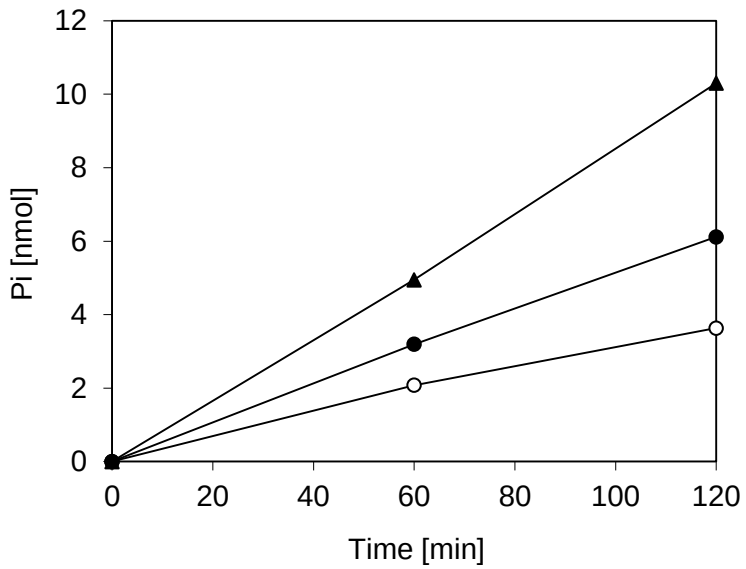

(a)
5
0.5
Absorption [600 nm]
0
1
2
3
CuSO4 [mM]
(b)
Wild-type
ΔcopA
-
Vector
Ec_copA
DOT_copA
DOT_copA2

Fig. 6


12
10
8
6
4
2
0
Pi [nmol]
0
20
40
60
80
100
120
Time [min]

Supplementary Material

Copper resistance and its regulation in the sulfate reducing bacterium *Desulfosporosinus* sp. OT

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Table S1. Primer sequences and use

Primer	5'-3' Sequence	Use
ha95	ATATGGCGCCTCACAAACTATCGACCTGACCCTGGAC	*Ec_copA* cloning
ha96	CCATTCTAGAGCGCATCCGCAATGATGTACTTATTCC	*Ec_copA* cloning
U1	GACAGGCGCCATGAATTCGGAAGAAAAAGTCCAC	*copU* cloning
U2	GACATCTAGACTCTCCTAAACGAGGCTTTTATTC	*copU* cloning
ha91	TATAGGCGCCACAAAACAAGCGATTCGTGAG	*DOT_copA* cloning
ha92	CGAGTCTAGAAACCCTATTGAAAGAACCCTAC	*DOT_copA* cloning
A21	GACAGGCGCCAAGGATCTTAGCCTACTTACG	*DOT_CopA2* cloning
A22	TATATCTAGACCATGACGGCCGACTG	*DOT_CopA2* cloning
ms109	TATAAGATGAAAGATTCATGTCATGG	EMSA
ms110	TCGCTATGGCAAAAAGATTC	EMSA
ms111	GACTTATAGTATAGGGGGGTATACTATATTAA	EMSA
ms112	TTAATATAGTATACCCCCCTATACTATAAGTC	EMSA
ms115	GAAGATTAGGTGCTTTAATACTAATATACTAAACTTGACTTATAGTATAGGGGGGTATACTATATTAATTAGCGAGGTGA	EMSA
ms116	TCACCTCGCTAATTAATATAGTATACCCCCCTATACTATAAGTCAAGTTTAGTATATTAGTATTAAAGCACCTAATCTTC	EMSA
sm143	ACATGAGTGGACTTTTTCTTC	EMSA
sm144	AGAGGGCGAACAGTAAAAAG	EMSA

Underlined nucleotides correspond to engineered NarI and XbaI enzyme recognition sites.

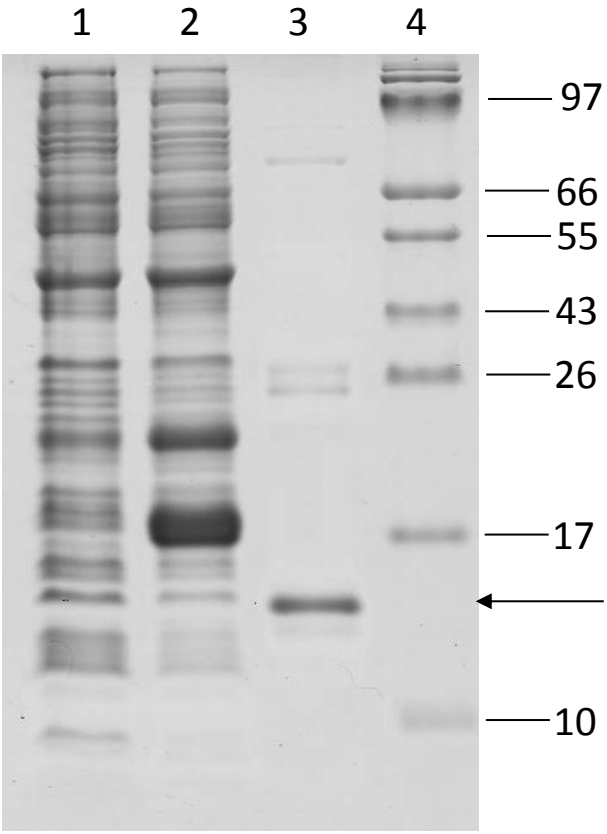

Fig. S1. Purification of CopU. *Lane 1*, 20 µg of crude cytoplasmic extract of uninduced BL21(DE3)RIL containing the CopU-expressing plasmid pOU2; *lane 2*, 20 µg of crude cytoplasmic extract 4 h after induction with 0.1 mM IPTG containing the overexpressed 6His-CopU; *lane 3*, 2 µg of purified CopU (predicted Mw = 12.8 kDa), indicated by the arrow; *lane 4*, molecular weight markers, with the sizes in kDa indicated on the right. Proteins were resolved on a 15 % tricine-SDS polyacrylamide gel and stained with Coomassie blue.

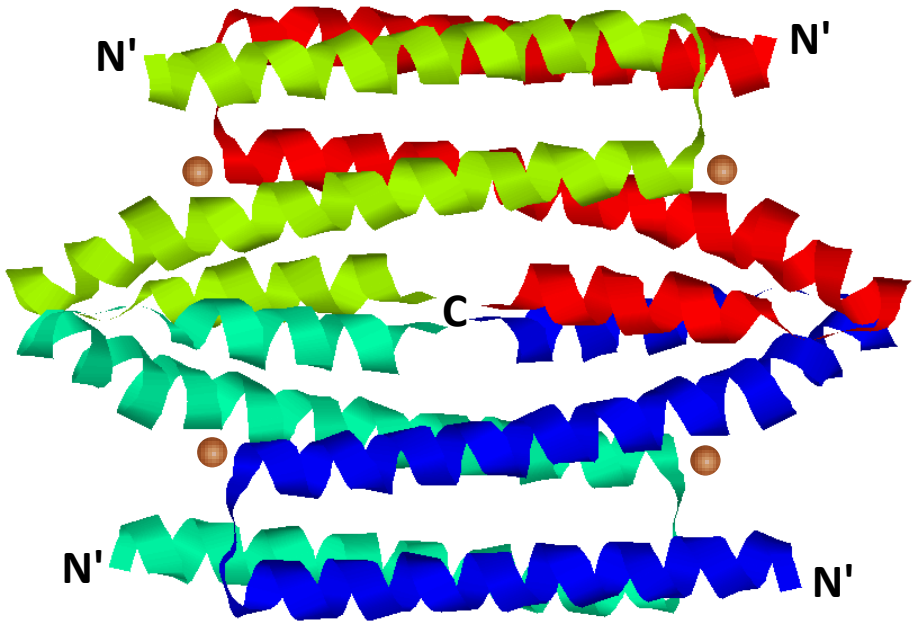

Fig. S2. Structural model of CopU. The structure of CopU was modeled with SWISS-MODEL (Biasini *et al.*, 2014). Each color represents a monomer of the homotetrameric structure. The modeled structure starts at H26 (N') and ends at the C-terminal residues (C). The brown spheres represent Cu(I) atoms which are predicted to be bound to H84 and C89 of one subunit and C59 of an adjacent subunit. The first 25 amino acids of CopU are missing in the model.

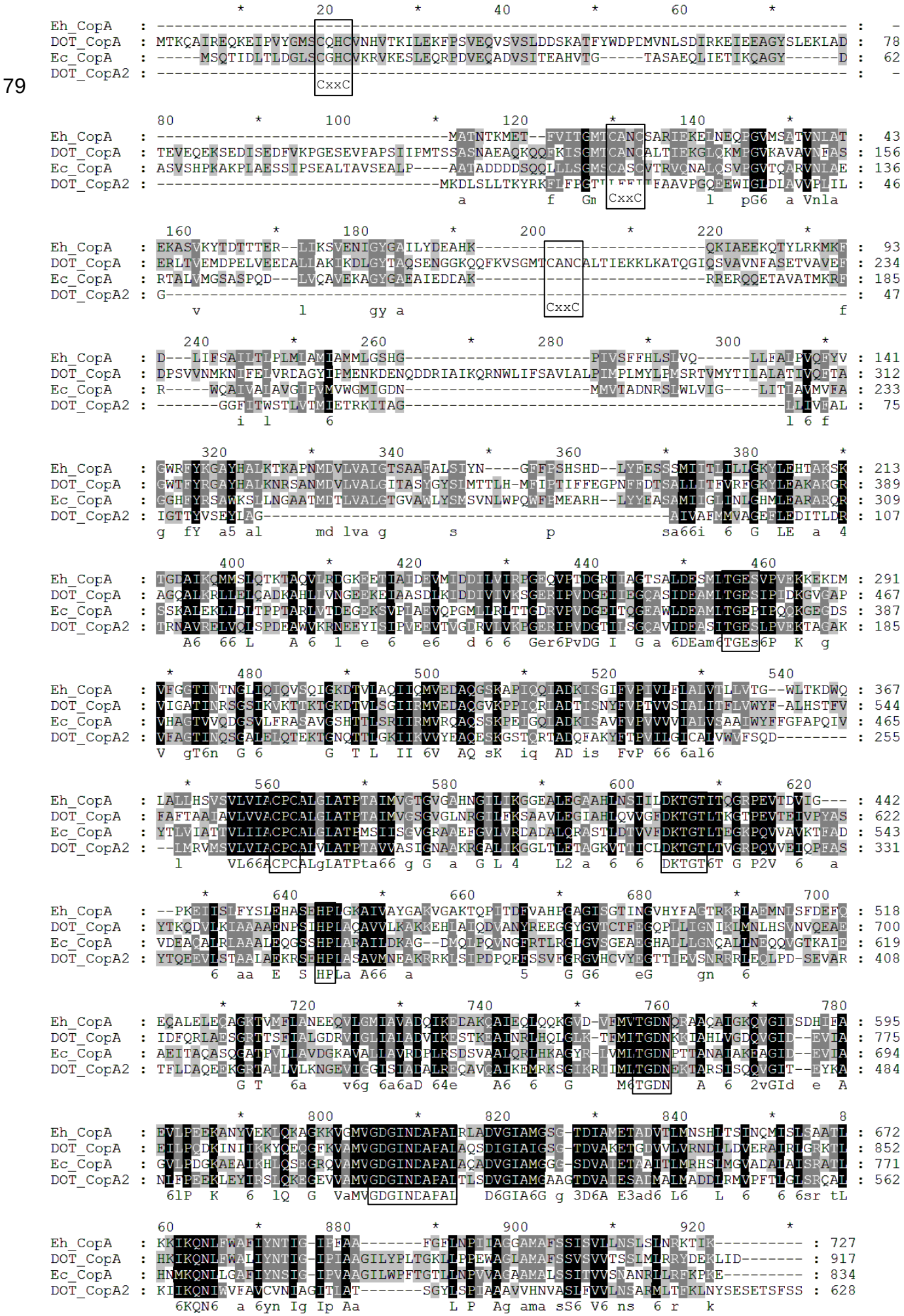
Eh_CopA
DOT_CopA
Ec_CopA
DOT_CopA2
CxxC
CxxC
CxxC
CxxC
CxxC

Fig. S3. Sequence alignment of ATPases Protein sequences were aligned with ClustalW. Conserved amino acids are displayed in inverse type and conservative replacements by a light- or dark-grey background. Eh_CopA (accession AAA61835); DOT_CopA (accession WP_009622712); Ec_CopA (accession BAE76263); DOT_CopA2 (accession number EGW36630). Conserved domains commonly found in P1B-ATPases are boxed. See main text for additional explanations.

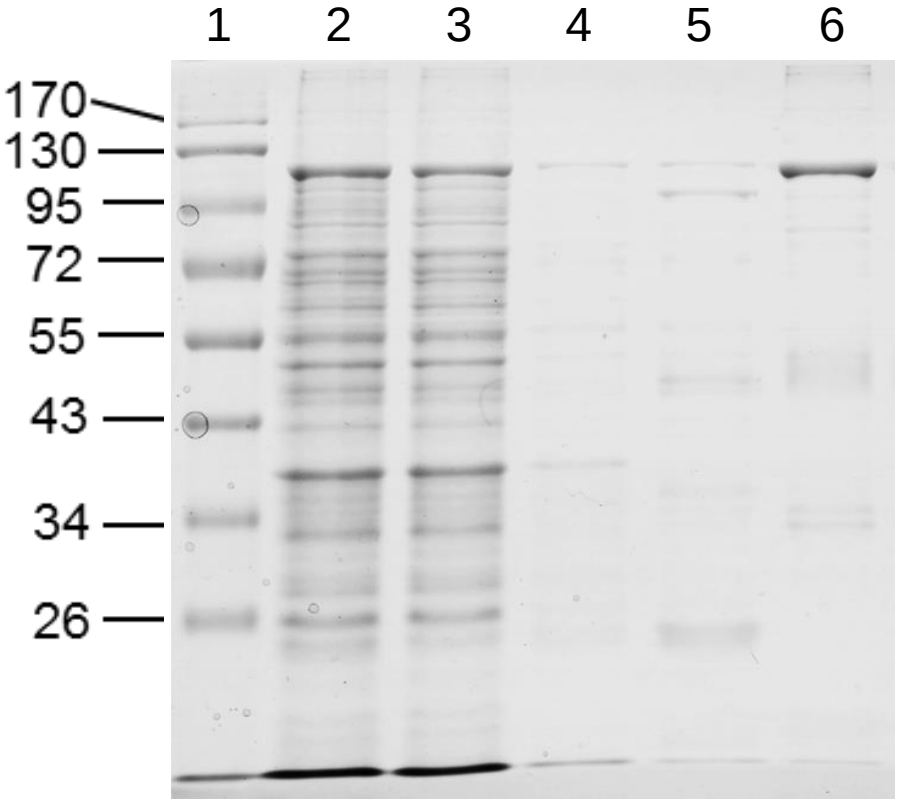

Fig. S4. Purification of DOT_CopA. Proteins were resolved on a 10 % glycine-SDS polyacrylamide gel and stained with Coomassie blue. *Lane 1*: Molecular weight marker with the sizes in kDa indicated on the left side of the Figure; *lane 2*: 15 μg solubilized membranes; *lane 3*: flow through of the Ni-NTA column; *lane 4:* wash with buffer JD; *lane 5*, elution with buffer JD + 200 mM imidazole; lane 6: 2 μg of purified DOT_CopA.